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The gene PRV-1 and its use

Description

The invention relates to a nucleotide sequence which encodes the PRV-1 gene, to recombinant DNA which contains this nucleotide sequence, to vectors which contain the recombinant DNA and to cells which are transformed with these vectors, and also to a PRV-1 polypeptide, to antibodies against this polypeptide, to a process for detecting the PRV-1 polypeptide and to drugs which comprise the PRV-1 polypeptide or antibodies which are directed against the PRV-1 polypeptide.

Polycythaemia rubra vera (erythraemia), also termed polycythaemia vera or p. vera, is a malignant haematological disease in which there is an increased formation of erythroid, granulocytic and megakaryocytic cells. The disease is of clonal origin and arises as a result of the mutation of a single haematopoietic precursor cell. In Germany, the incidence of p. vera is from 4 to 6 per million inhabitants. If left untreated, the disease leads to death within 18 months. Treatment by means of blood-letting or chemotherapy extends the average survival time to more than 13 years.

P. vera is diagnosed by means of clinical criteria. The clinical picture includes headaches, pruritus, splenomegaly in two thirds of the patients, bleeding or thromboses, hypertension in a third of the patients, gout, which is brought about by an increase in the production of uric acid, and, in some cases, septic ulcers. The most important laboratory finding is an increase in the values for haemoglobin, haematocrit, erythrocyte count and total erythrocyte volume, and also a neutrophilic granulocytosis or thrombocytosis in

many cases. Since, on the one hand, most of the criteria are rather diffuse and, on the other hand, not all the patients fulfil these criteria, it is frequently difficult to distinguish p. vera from other myeloproliferative diseases, such as chronic granulocytic leukaemia or essential thrombocytosis, and thereby confirm the diagnosis. To date, the molecular cause of p. vera is completely unknown. Since, however, p. vera takes a severe course if it is not treated, accurate diagnosis is important.

An object of the invention was therefore to find the molecular cause of polycythaemia rubra vera and to create the possibility of diagnosing it.

This object was achieved by isolating a gene which is expressed specifically in association with p. vera and not in healthy control individuals. This gene is designated the PRV-1 gene (polycythaemia rubra vera).

A similar nucleotide sequence is disclosed in International application WO 98/50552.

One part of the subject-matter of the invention therefore relates to a polynucleotide which encodes the PRV-1 gene and essentially comprises the sequence ID No. 1. The polynucleotides of the present invention can be single-stranded or double-stranded DNA or RNA. If they are RNA, it is then clear to the skilled person that "U" nucleotides are present in place of "T" nucleotides. "Polynucleotide" is understood as meaning nucleic acids which contain 15 or more nucleotides.

The nucleotide sequence according to the invention is depicted in Figure 1. The invention therefore relates to a polynucleotide which corresponds to the sequence shown in Figure 1 and also to a polynucleotide whose nucleotide sequence exhibits minor differences. Within the meaning of the present application, minor differences are understood as meaning those sequences in which a few, preferably not more than 50 and particularly preferably not more than 25, nucleotides can be exchanged, with, however, the

function of the gene encoded by the nucleotide sequence being unaffected. The skilled person is familiar with the fact that a base triplet encoding an amino acid can be replaced with another triplet which encodes the same amino acid. In addition to this, regions which are of less importance can be deleted and/or mutated to a minor extent. In a particular embodiment, the polynucleotide comprises nucleotides 36 to 1346 of sequence No. 1, that is the coding region of the PRV-1 gene. Another embodiment comprises nucleotides 36 to 1262 of sequence No. 1. This region presumably encodes the active region of the PRV-1 polypeptide. Finally, the polynucleotide of the invention can also comprise nucleotides 39 to 1346 or 39 to 1262 of sequence No. 1, such that the codon which encodes the starting methionine is not present. A preferred embodiment is a polynucleotide which comprises nucleotides 99-1346 or 99 to 1262 of sequence No. 1. This results in the codons at the 5′ end which encode the signal peptide of the PRV-1 polypeptide not being present.

The polynucleotide according to the invention can also be a fragment of the PRV-1 gene. As a rule, the fragment possesses more than 100 nucleotides, preferably, however, more than 300 nucleotides. The fragments can also be used as primers or as probes, in particular for PCR; in this case, the fragments can be truncated to fit the purpose. Usually, primers have a length of between 10 and 30 nucleotides and probes have a length of between 15 and 50 nucleotides.

The PRV-1 gene is an endogenous gene whose expression in healthy individuals is, however, restricted to only a few organs. Normally, it is expressed in the main in the haematopoietic organs, i.e. in bone marrow and foetal liver, and weakly expressed in the spleen, but not expressed in heart, muscle, pancreas or kidney. In patients who are suffering from p. vera, this gene is very strongly overexpressed in the haematopoietic cells, in particular.

The PRV-1 gene encodes a protein which exhibits the protein sequence shown in Figure 2. The signal peptide, which is present in the protein sequence of all surface molecules and normally removed when the protein is processed, is divided off by a hyphen. The protein has the sequence ID No. 2. Another aspect of the invention is consequently an essentially pure polypeptide having the sequence No. 2 or a polypeptide having the sequence No. 2 but lacking the signal peptide (i.e. amino acids 22 to 437 of sequence No. 2). Other embodiments encompass amino acids 1 to 409 or 22 to 409 of sequence No. 2 (what is probably the active region of the protein).

With regard to biological activity, the polypeptide according to the invention is preferably glycosylated; it is most preferably N-glycosylated. It can then be glycosylated at at least one of the amino acids Asn-46, Asn-189 and Asn-382 of the PRV-1 polypeptide (the amino acid numbers refer to the sequence No. 2). The invention also encompasses fragments of the polypeptides according to the invention which are N-glycosylated. The fragments are at least 50 amino acids in length, preferably at least 100 amino acids and most preferably at least 150 amino acids. In another embodiment, a polypeptide can be O-glycosylated.

It is clear to the skilled person that particular amino acids can be replaced with other amino acids without impairing the biological activity of the protein. Such modified forms of the polypeptides according to the invention are also part of the subject-matter of the invention. The amino acid replacements are those which do not have a negative effect on the biological activity of the protein. The skilled person can make use of well known rules for selecting the replacements.

Depending on the method of preparation, the PRV-1 polypeptide can, for example, possess a glycosyl phosphatidylinositol anchor. This is then bonded to the

amino acids which correspond to amino acids 407 to 409 in sequence ID No. 2. A GPI anchor is used to anchor a protein by means of a lipid on the outside of the cell membrane. However, for reasons which have not so far been conclusively elucidated, it is frequently observed that GPI-linked proteins are also released into the medium. This is referred to as "shedding". To date, it has not been clarified whether this is a specific process, i.e. such proteins are cleaved from the membrane by enzymes in a controlled manner, or whether it represents a non-specific loss of the anchor. It is consequently very probable that PRV-1 is to be found both on the cell membrane and extracellularly. The secreted form, which is not membrane-bound, is probably more important for the effect of the polypeptide as a growth factor since, as a growth factor, this form is able to diffuse and reach other cells.

It is clear to the skilled person that he can influence the attachment of the protein to the cell membrane by manipulating these amino acids. This particularly concerns the preparation of defined DNA constructs which are intended for expressing the PRV-1 polypeptide or fragments of this polypeptide. The codons which encode these amino acids can be mutated or deleted.

The gene encodes a surface receptor of the uPAR/Ly6 family. This receptor family can transduce mitogenic signals, i.e. signals which stimulate cell division. It is therefore assumed that overexpression of the PRV-1 gene, inter alia on the granulocytes of p. vera patients, contributes to hyperproliferation of these cells.

It has been found that PRV-1 is not expressed on granulocytes in healthy individuals or in patients suffering from other myeloproliferative diseases, e.g. suffering from chronic granulocytic leukaemia, acute granulocytic leukaemia or essential thrombocytosis.

In order to be able to use the polypeptide encoded by the PRV-1 gene for analyses and detection

methods, it is expediently generated from recombinant DNA, with the recombinant DNA preferably comprising the nucleotide sequence ID No. 1 or at least the coding region of the PRV-1 gene, that is nucleotides 36 to 1346 of sequence ID No. 1, at least, however, nucleotides 39 to 1262, functionally linked to a promoter. However, the recombinant DNA can also comprise only a fragment of sequence No. 1.

The invention furthermore relates to a vector which contains the recombinant DNA for the PRV-1 polypeptide, or a fragment thereof, and to a host cell which is transfected or transformed with this vector. The host cells may be prokaryotic, for example bacteria such as E. coli. However, the polypeptides which are expressed are then not glycosylated. Preference is therefore given to eukaryotic host cells, which are able to glycosylate the expressed protein post-translationally and modify it in other ways. Examples of eukaryotic host cells are insect cells, such as Sf9 cells, for expression following infection with recombinant baculoviruses, and mammalian cells, such as COS cells, CHO cells and HeLa cells. These examples are not exhaustive. It is also possible to use yeast cells as host cells. It is clear to the skilled person that the glycosylation pattern can differ depending on the host cell. The biological activity of the expression product can therefore also vary. Particular preference is given to host cells which glycosylate the expression product in such a way that the biological activity of the protein is retained.

The PRV-1 polypeptide which is isolated from granulocytes or produced recombinantly can be employed both for diagnosing polycythaemia vera and for treating the disease.

One therapeutic possibility is that of "antisense therapy". This method employs an "antisense" RNA molecule, that is an RNA which is complementary to the PRV RNA. Since the PRV-1 RNA has the sequence 5'-AAAAGCAGAAAGAGATTACCAGCC-3' (seq. ID No. 3) at its

beginning, the requisite antisense RNA directed against this sequence would possess the following nucleotide sequence: 5'-GGCTGGTAATCTCTTTCTGCTTTT-3' (seq. ID No. 4). This antisense RNA is incorporated into a vector and introduced into the p. vera cells. This RNA is introduced, for example, by means of transfection, with the vector used for the transfection preferably being configured such that it is introduced specifically into the p. vera cells. Expression of the antisense RNA results in it no longer being possible for the PRV-1 mRNA to be translated into a polypeptide. Cells which have been treated in this way do not then form any PRV-1 protein.

The invention therefore also relates to a process for detecting p. vera which is characterized in that the PRV-1 polypeptide, or an epitope thereof, is detected and the extent of the expression is determined.

Overexpression of this receptor on mature cells outside of the bone marrow, e.g. on granulocytes, is a strong indication of the presence of the disease p. vera. This overexpression is expediently detected by means of an immunoassay using antibodies which are directed against the PRV-1 receptor. Suitable test methods are the known immunoassay variants which make use of PRV-1 polypeptide-specific antibodies together with other labelled antibodies which can be immobilized or in solution. The labelling can be effected in a manner known per se, for example using radioactive isotopes, by means of fluorescence or luminescence, using enzymes, by means of colour-forming reactions or using other groups which are suitable for the determination. These variants are known to the skilled person and do not require any more detailed explanation here. According to the invention, ELISA tests are particularly preferred.

The antibodies which are required for specifically detecting the PRV-1 receptor can likewise be prepared in a manner which is known per se. Both

monoclonal and polyclonal antibodies are suitable, with preference being given to using monoclonal antibodies.

Peptides which are derived from the protein can also be used for preparing antibodies. Within the context of the present invention, success was achieved using the peptides having the sequences:

a) KVSDLPRQWTPKN (amino acids 34 to 46) [seq. ID No. 5], and

b) SAREKRDVQPPASQH (amino acids 391 to 405) [seq. ID No. 6].

The polyclonal antibodies are normally produced by immunizing a suitable host (rabbit) with the PRV-1 polypeptide, where appropriate bound to an immunological support (adjuvant), and eliciting an immune response. Monoclonal antibodies can be generated in a manner known per se using the hybridoma technique. The antibodies can be purified by means of affinity purification. The preparation and purification of antibodies are described, for example, in "Antibodies: A Laboratory Manual" by Harlow and Lane, Cold Spring Harbor Laboratory Press.

Furthermore, such polyclonal or monoclonal antibodies which are directed against PRV-1 can also be used for treating the disease.

In another embodiment, the PRV-1 receptor can be detected using an RT-PCR method. For this, RNA is first of all isolated from the PRV-1-overexpressing cells, which are as a rule granulocytes. A reverse transcription is then performed in a manner known per se using an RT primer. The RT primer is preferably a primer which has the following nucleotide sequence (SEQ ID No. 7):

ATTAGGTTATGAGGTCAGAGGGAGGTT.

In this way, the specific PRV-1 RNA is transformed into DNA. This DNA is then amplified in a PCR reaction in a manner known per se. The following two primers are preferably employed for the amplification cycles:

As the sense primer (SEQ ID No. 8)

GCAGAAAGAGATTACCAGCCACAGACGG.

As the antisense primer (SEQ ID No. 9)

GAATCGTGGGGGTAATAGAGTTAGCAGG.

The skilled person is readily able to use the disclosed sequence to find other primers which are also suitable.

Since the RNA is used as the starting material for this method, the PCR signal is only positive when the PRV-1 gene is also expressed. As explained above, this is only the case when the patient is suffering from p. vera. PRV is not expressed in granulocytes of healthy patients. Consequently, the absence of any RT-PCR signal indicates that no p. vera is present.

In another alternative, it is also possible to use a blotting method, preferably a Northern Blot, for diagnosing p. vera. For such a method, the RNA is isolated from granulocytes and then examined for the expression of PRV-1 using a blotting method, for example Northern blotting. The cDNA sequence of SEQ ID No. 1, or a segment of the sequence, can be used as the probe. Hybridization then only occurs if the granulocytes are derived from a patient suffering from p. vera since only then is there any expression on the granulocytes. The absence of hybridization indicates that the individual from whom the granulocytes are derived does not have p. vera.

It is also possible to use a fragment of the gene for the Northern blot hybridization. Such a

fragment is normally more than 100 bases in length, preferably more than 300 bases in length. Alternatively, various different fragments of the gene, which can be used as probes in the Northern blot, can be prepared by digesting the gene with restriction endonucleases. If the fragments are derived from the cDNA, they are then present as double strands which have to be separated into the single strands for the hybridization. Suitable examples are the Bam HI-PstI fragment from base pair 420 to base pair 831, or the PstI-PstI fragment from base pair 831 to base pair 1900.

PRV-1 mRNA, and consequently the expression of PRV-1, can also be detected by first of all reverse-transcribing the mRNA in an RT-PCR reaction and then amplifying the cDNA; the amplified DNA is then detected with a probe in a hybridization method.

In the case of a positive diagnosis, the disease has to be treated since it otherwise leads to death within a relatively short period of time. For this treatment, it is possible to use specific antibodies which are directed against PRV-1 and to which cytotoxic components can be bonded, where appropriate.

The invention therefore furthermore relates to a drug which, in addition to the customary excipients, comprises antibodies which are directed against the PRV-1 receptor.

Since the PRV-1 receptor is overexpressed in p. vera, many antibodies are bound on the surface of the affected granulocytes when they come into contact with the anti-PRV-1 antibody. The binding of many antibodies to these cells stimulates the immunological cells to destroy these granulocytes. In this way, it is possible to eliminate the p. vera cells specifically.

Surprisingly, it has also been found that the PRV-1 polypeptide exhibits haematopoietic activity. The PRV-1 polypeptide is able to stimulate certain haematopoietic precursor cells to form erythroid

colonies. It is particularly the N-glycosylated PRV-1 polypeptides which display this function. The polypeptides according to the invention which are preferred are therefore the N-glycosylated PRV-1 polypeptides, and fragments thereof, which display the growth factor activity.

Another aspect of the invention is therefore a drug which, in addition to a pharmaceutically tolerated excipient, comprises the PRV-1 polypeptide or a biologically active fragment thereof. The PRV-1 polypeptide is preferably glycosylated PRV-1 polypeptide and, even more preferably, N-glycosylated PRV-1 polypeptide or a biologically active fragment thereof. The invention also relates to drugs which comprise at least one polynucleotide according to the invention.

The present invention furthermore relates to the use of PRV-1 polypeptide, or a biologically active fragment thereof, as a growth factor in vivo and ex vivo. The PRV-1 polypeptide, or a biologically active fragment thereof, can be used for treating all pancytopenias and pancytopathies in the bone marrow and in the circulation (change in the cellular constituents of the peripheral blood and bone marrow). The polypeptides of the present invention can, for example, be used for treating anaemias in the case of kidney failure, chemotherapy or whole body radiation, for treating neutropenias and thrombocytopenias during chemotherapy or whole body radiation, for the ex-vivo treatment of peripheral or bone marrow stem cells for expansion (multiplication) and retransfusion into the patients, and for treating sepsis, systemic inflammatory response syndrome (SIRS) or regional inflammatory reactions. The polypeptides of the present invention, or drugs which comprise them, can be administered in a wide variety of ways. The forms of administration comprise intravenous, intramuscular, subcutaneous, intraperitoneal, oral, transdermal and transmucosal administration.

The polynucleotides according to the invention can also be used for treating pancytopenias and pancytopathies. In this case, the aim is to express a PRV-1 polypeptide, or a functional fragment thereof, in cells of the affected patient. Gene therapy methods are first and foremost used in this connection. Cells can be isolated from the patient and transfected with a polynucleotide according to the invention (ex-vivo manipulation), after which they are then returned to the patient. It is also possible to conceive of methods in which the polynucleotides according to the invention gain access into the target cells by means of viral transfer. Expression of the inserted nucleic acids then leads to haematopoietic activity.

The invention also relates to kits for detecting either polycythaemia vera or disturbances of the haematopoietic system. These kits comprise a polynucleotide according to the invention and/or a polypeptide according to the invention and/or one or more antibodies according to the invention. In addition to this, the kit can also comprise a container or compositions which are suitable for implementing detection reactions. Examples of such compositions are buffer solutions, reagents for blocking membranes, hybridization solutions, secondary antibodies, substrate solutions for detection reactions, etc. The kit is preferably used for implementing PCR reactions, Northern blots, Southern blots, Western blots and ELISA, RIA or similar reactions.

The following examples are given in explanation.

Example 1

Characterizing the PRV gene

The following experiments were carried out in order to characterize the gene:

- the following protocol was used to isolate granulocytes from stored blood or from blood obtained by bleeding p. vera patients:

- an equal volume of 3% dextran solution in 0.9% NaCl was added to the blood and the mixture was left to stand at room temperature (RT) for 20 minutes.

- The mixture separated into two phases. The upper, light-coloured phase was removed and centrifuged for 10 minutes at 1800 g and at RT.

- The supernatant was discarded and the cell pellet was resuspended in the same volume of 0.9% NaCl.

- In each case 35 ml of the cells in NaCl were layered on 15 ml of Ficoll-Hypaque.

- The cells on the Ficoll-Hypaque were then centrifuged for 60 minutes at 1800 g and at RT without using the brake.

- A cell pellet and two layers with an interphase were formed.

- The layers and interphase were aspirated off and the cell pellet was resuspended for 30 seconds in 10 ml of ice-cold 0.2% NaCl, and 10 ml of ice-cold 1.6% NaCl were added immediately after 30 seconds.

- The cells were centrifuged down for 10 minutes at 1800 g and at RT.

- They were then washed once in 10 ml of PBS and centrifuged down.

- The cell pellet contained 95-99%-pure granulocytes.

- RNA was isolated from these cells using standard methods.

- 10 mg of this RNA were examined for the expression of PRV-1 in a Northern blot. The entire cDNA sequence shown in SEQ ID No. 1 was used as a probe.

This experiment was performed on 19 p. vera patients and 21 control samples of stored blood. The PRV-1 probe was found to hybridize strongly in the case of the p. vera patients. No hybridization was observed in healthy control samples.

Example 2

PRV-1 possesses growth factor activity

Embryos were removed from a pregnant mouse 13.5 days after fertilization. The foetal livers were removed. The cells contained in them were stained using antibodies and enriched for particular cells, and depleted for other cell types, by means of column chromatography. This results in a cell mixture which is enriched for certain haematopoietic precursor cells (colony forming units-erythroid, CFU-E). Thus, while in all approximately 2% of the foetal liver consists of CFU-E, 30-40% of the enriched cells consist of CFU-E.

These CFU-Es were transfected using a retrovirus. To do this, a packaging cell line, designated 293-T, was itself transfected 48 hours previously. 293-T cells are an established human embryonic kidney cell line. 293-T cells are stably transfected with several genes from a retrovirus. If these 293-T cells are now transfected with two plasmids, termed pOS and pKAT, the 293-T cells then produce a retrovirus which is able to infect murine foetal liver cells. If the 293-T cells are transfected with an empty pOS vector and pKAT, a wild-type retrovirus, which only expresses retroviral proteins, is then produced. On the other hand, cloning a human gene, e.g. PRV-1, into the pOS vector results in the production of a retrovirus which expresses this protein when it has infected cells. The 293-T cells secrete the retrovirus into the cell culture medium.

After two days, the cell culture medium from the transfected 293-T cells which contains the retrovirus is harvested and filtered once through a 0.45 μm filter. In order to transfect the foetal liver cells, these latter cells are mixed with the filtered cell culture medium, which contains the retrovirus, and centrifuged for 2 hours at 1800 rpm and 20°C in the added presence of Polybren. The transfected foetal

liver cells were then cultured in a medium (Methocult, from Cell Systems) which contains, in addition to the usual salts and amino acids, foetal calf serum, 0.0001-0.4 IU of erythropoeitin (EPO)/ml and methyl cellulose (0.8%). The CFU-Es require EPO in order to form haematopoietic colonies. The methyl cellulose solidifies the medium in the form of a jelly, thereby fixing individual cells in this jelly so that, in contrast to being in a liquid medium, they cannot move. It is therefore possible to observe whether a haematopoietic colony is or is not formed from a single cell. CFU-Es form erythroid colonies, that is colonies which contain red blood cells and their precursor cells.

After three days, a count is taken of the number of haematopoietic colonies which have developed. Various mixtures are compared. The mixtures were not all examined in each experiment; mixtures 1-3 are very similar controls and each of them can be compared individually with mixture 4.

Mixture 1: Cells which were not transfected with a retrovirus;

Mixture 2: Cells which were transfected with an empty pOS vector;

Mixture 3: Cells which were transfected with a "green fluorescent protein" (GFP), a protein which is not haematopoietically active.

Mixture 4: Cells which were transfected with pOS-PRV-1 (vector + gene according to the invention).

Table 1: The table lists the results obtained from three experiments which were performed as described. The figures in each case indicate the number of colonies.

	Mixture 1	Mixture 2	Mixture 3	Mixture 4
	un-transfected	empty vector (pOS)	GFP (pOS-GFP)	PRV-1 (pOS-PRV-1)
Experiment 1	116	156	80	326
Experiment 2		271	273	410
Experiment 3	120		131	291

The experiments demonstrate that CFU-Es which were transfected with PRV-1 form very many more colonies (up to three times as many) than do the various control CFU-Es. This result indicates that PRV-1 is a growth factor for CFU-E.

Example 3

Solubility of the PRV-1 growth factor

A further experiment was carried out in order to investigate whether PRV-1 is a soluble growth factor or whether cell-cell contact is required. It is not only a retrovirus which is produced by the packaging cell line 293-T after it has been transfected with the pOS and pKAT vectors. In addition, the 293-T cells also synthesize the protein encoded by the gene cloned in pOS, i.e. PRV-1 in the present case. If the gene product is a soluble protein, it is secreted into the medium which surrounds the packaging cell line 293-T. If the 293-T cells are transfected only with the pOS vector, without pKAT, no retroviruses are then formed. The cell culture medium then only contains the soluble protein produced by the cells. Medium which is derived from pOS-PRV-1-transfected cells, and which does not contain any retrovirus, is mixed with CFU-Es and the whole is plated out in the methyl cellulose medium; the resulting colonies are then counted.

The following results were obtained:

Table 2: Solubility of PRV-1. The figures in each case indicate the number of colonies.

	Mixture 1	Mixture 2	Mixture 3	Mixture 4
	un-transfected	empty vector (pOS)	GFP (pOS-GFP)	PRV-1 (pOS-PRV-1)
Experiment 4		137	187	557

In this experiment, too, CFU-Es which were treated with PRV-1-containing medium formed very many more haematopoietic colonies than did control cells. It can be concluded from this result that PRV-1 is a soluble growth factor.

Example 4

The growth factor PRV-1 is N-glycosylated

Granulocytes were isolated from a patient suffering from p. vera, and protein extracts were prepared from these cells using a standard protocol. These protein extracts were treated in accordance with the protocol for the "N-Glycosidase F Deglycosylation Kit" supplied by Boehringer Mannheim. In detail, this means that a "denaturation buffer" was added to the protein extracts and the mixtures were heated at 95°C for 3 minutes, after which they were treated either with "reaction buffer" or with "reaction buffer" plus N-glycosidase. Each mixture was incubated overnight at 37°C and the proteins were analysed on a PAGE gel electrophoresis followed by a Western blot. The PRV-1 protein was detected with an antibody directed against a protein having the amino acid sequence ID No. 5. The results show that while PRV-1 protein purified from granulocytes is 60-65 kDa in size, it is only 40 kDa in size after having been digested with N-glycosidase. This clearly proves that PRV-1 is glycosylated on asparagine residues (asparagine = N).